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Simultaneous detection of the C282Y, H63D and S65C mutations in the hemochromatosis gene using quenched-FRET real-time PCR

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Hereditary hemochromatosis (HH) is a common autosomal disorder of iron metabolism mainly affecting Caucasian populations. Three recurrent disease-associated mutations have been detected in the hemochromatosis gene (*HFE*): C282Y, H63D, and S65C. Although HH phenotype has been associated with all three mutations, C282Y is considered the most relevant mutation responsible for hemochromatosis. Clinical complications of HH include cirrhosis of the liver, congestive cardiac failure and cardiac arrhythmias, endocrine pancreatic disease, which can be prevented by early diagnosis and treatment. Therefore, a reliable genotyping method is required for presymptomatic diagnosis. We describe the simultaneous detection of the C282Y, H63D and S65C mutations in the hemochromatosis gene by real-time PCR followed by melting curve analysis using fluorescence resonance energy transfer (FRET) probes. The acceptor fluorophore may be replaced by a quencher, increasing multiplex possibilities. Real-time PCR results were compared to the results of sequencing and conventional PCR followed by restriction digestion and detection by agarose gel electrophoresis (PCR-RFLP). Genotypes from 80 individuals obtained both by the conventional PCR-RFLP method and quenched-FRET real-time PCR were in full agreement. Sequencing also confirmed the results obtained by the new method, which proved to be an accurate, rapid and cost-effective diagnostic assay. Our findings demonstrate the usefulness of real-time PCR for the simultaneous detection of mutations in the *HFE* gene, which allows a reduction of a significant amount of time in sample processing compared to the PCR-RFLP method, eliminates the use of toxic reagents, reduces the risk of contamination in the laboratory, and enables full process automation.

Key words: Hemochromatosis; Single nucleotide polymorphism; Quenched-FRET; Real-time PCR

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HFE-related hereditary hemochromatosis (HH) is an autosomal disorder of iron metabolism causing iron overload status and affecting Caucasian populations with a prevalence of approximately 1 in 300 (1). Clinical consequences of iron overload include cirrhosis of the liver, congestive cardiac failure and cardiac arrhythmias, and endocrine pancreatic disease, which can be easily prevented by early diagnosis and iron depletion by phlebotomy. Three recurrent missense mutations in the *HFE* gene have been associated with HH: C282Y, a cysteine-to-tyrosine amino acid substitution caused by an 845 G>A transition at codon 282 within exon 4; H63D, a 187 C>G transversion within exon 2 that results in a change at codon 63 from histidine to aspartate, and S65C, a serine-to-cysteine substitution generated by a 193 A>T transversion in exon 2. C282Y has been well characterized as

being the main mutation responsible for hemochromatosis in all populations studied. Even though most individuals with HH are homozygous for the mutation C282Y (81-90%), a small number of compound heterozygous for C282Y and H63D may also develop clinical iron overload (2). The mutation S65C arises in 2-3% of Caucasians and is implicated in mild iron overload when inherited in the compound heterozygous state with either C282Y or H63D (3,4).

Methods currently available for routine genotyping of HH patients include polymerase chain reaction restriction fragment length polymorphism analysis (PCR-RFLP); PCR-mediated site-directed mutagenesis (5); oligonucleotide ligation; reverse hybridization line-probe assay (LiPA) (6); sequencing; allele-specific PCR (7), and real-time PCR with fluorescence resonance energy transfer (FRET) probes followed by melting analysis in the LightCycler™ (Roche Applied Science, Germany) (8,9). Conventional real-time PCR with FRET probes (also called hybridization probes) employs two labeled oligonucleotide probes, usually named FRET and anchor probes, that bind to the PCR product in a head-to-tail fashion. One of these probes is labeled with a donor dye at the 3' end and the other is labeled with an acceptor dye at the 5' end. As the probes hybridize to adjacent regions in the same strand, their fluorophores come into close proximity, which allows energy transfer from a donor to an acceptor fluorophore. The acceptor fluorophore then emits light in a longer wavelength, which is used for signal detection. Traditionally, FRET experiments have been performed in the LightCycler instrument, where LightCycler®-Red 640 (LC-Red 640) and/or LightCycler®-Red 705 (LC-Red 705) are the dyes usually employed as acceptor fluorophores. The requirement of a spectral overlap of donor emission and acceptor excitation results in an overlap of the emission bands, since it is a general feature of fluorescent dyes that they exhibit broad emission spectra. This cross-talk must be compensated in dual-color experiments by a color-compensation calibration (10).

Quenched-FRET assays are similar to FRET assays, differing by the fact that the decrease in energy of the donor fluorophore during the amplification is measured instead of the increase in energy of the acceptor fluorophore. Use of a quencher molecule, such as black-hole quenchers, in place of an acceptor fluorophore enables multiplexing of more than one fluorophore, permitting the analysis of a greater number of mutations in the same tube. Since quenched-FRET makes use of less spectral bandwidth per probe set, there is no need to use expensive proprietary dyes to minimize cross-talk for multiplex applications or to perform color-compensation calibration. As FRET probes are not hydrolyzed, genotyping is achieved by performing a melting analysis after PCR for the determination of the amplicon-probe melting temperatures, which may show variation depending on the number of mismatches, the length of the mismatched duplex, the position of the mismatch, and neighboring base pairs. The increase in energy of the donor probe is measured during melting analysis. This technique was successfully employed for factor V Leiden and prothrombin genotyping on the Rotor-Gene 2000 (11).

Here we describe a new diagnostic assay based on quenched-FRET real-time PCR for the simultaneous detection of the C282Y, H63D and S65C mutations in *HFE* using the Rotor-Gene 3000 (Corbett Research, Australia).

Peripheral blood was collected from 80 individuals (60 men, mean age 49 years, range 12-75 years; 20 women, mean age 49 years, range 6-75 years) who attended Fleury Medicina e Saúde for *HFE* genotyping after medical indication. This study was approved by the Institutional Ethics Committee. Genomic DNA was extracted using QIAGEN BioRobot 9604 (QIAGEN, Germany) in a fully automated process.

Multiplex PCR in the Rotor-Gene used two unlabeled primer sets for amplification and two pairs of fluorescent-labeled probes for detection of each single nucleotide polymorphism (SNP). Primers and probes for C282Y and H63D were designed using the Beacon Designer software (Premier Biosoft International, USA; Table 1) based on the *HFE* gene reference sequence (GenBank NM_139011). For the detection of the S65C mutation, the same primers and probes designed for H63D were employed, as these two SNPs are only six nucleotides apart and the detector probe for codon 63 overlaps the polymorphic position at codon 65. The C282Y FRET probe was designed to match the mutated allele and labeled with 6-FAM. The H63D/S65C FRET probe was designed to be complementary to the mutated/wild-type allele, respectively, and labeled with a different fluorophore (JOE).

The mutation was detected by real-time PCR followed by melting curve analysis with adjacent fluorescent probes using the quenched-FRET principle. FRET and anchor probes hybridize just a few nucleotides apart (five nucleotides for C282Y probes and three nucleotides for H63D probes).

For each sample, 120 ng genomic DNA in a final PCR volume of 25 µL was amplified and detected in the Rotor-Gene 3000. For standardization, multiplexed amplification reactions were performed using distinct Master Mixes and different concentrations of primers and probes to test for the best combination. Cycling and melting profiles were performed according to the following protocol: 95°C

for 4 min as the initial denaturation step, followed by 50 cycles of 95°C for 10 s, 53°C for 20 s, and 72°C for 20 s. Thereafter, melting curve analysis of the duplex amplicon-probe was performed, starting from 49°C and proceeding until 88°C, at a linear rate of 1°C every 5 s.

For all samples, genotyping performed in the Rotor-Gene 3000 was compared to conventional PCR restriction fragment analysis for both the C282Y and H63D mutations. Conventional multiplex PCR was performed using the primers C282YP1 (5' TGGCAAGGGTAAACAGATCC 3'), C282YP2 (5' CTCAGGCACTCCTCTCAACC 3'), H63DP3 (5' CCAGACACAGCTGATGGTAT 3') and H63DP2 (5' GCCACATCTGGCTTGAAATT 3'). The amplicons were digested with the endonucleases *Rsa*I and *Bcl*I for detection of the mutations C282Y and H63D, respectively. The C282Y creates a new *Rsa*I site, whereas the H63D mutation abolishes the *Bcl*I recognition site in the PCR product. Products of digestion were submitted to electrophoresis on 4% Nusieve gels for 1 h at 100 W and visualized with UV light after staining with ethidium bromide.

For automated sequencing, PCR fragments were prepurified on GFX columns (GE Healthcare Life Sciences, USA) according to manufacturer instructions. Sequencing reactions were carried out using BigDye Terminator v3.1 (Applied Biosystems, USA) and the same primers employed for amplification by real-time PCR. The sequencing products were run on the ABI 3130xl Prism Genetic Analyzer (Applied Biosystems) and analyzed using the software BioEdit Sequence Alignment Editor (Ibis Biosciences, USA). Sequences obtained were compared to the *HFE* gene reference sequence.

The best results, considering resolution, discrimination of peaks and the amplitude of the maximum normalized fluorescence signal during melting analysis, were obtained under the following conditions: 1X Promega PCR Master Mix (1.5 mM $MgCl_2$, 200 µM each dNTP, 0.6 U *Taq* DNA polymerase); 0.06 µM each forward primer (C282Y-F and H63D-F); 0.2 µM each reverse primer (C282Y-R and H63D-R); 0.2 µM each probe (C282Y and H63D, both anchor and FRET). As expected, asymmetric PCR resulting in the production of greater amounts of target strands significantly increased the efficiency of allelic discrimination. Typical FRET melting profiles are shown in Figure 1. Figure 1A shows three curves corresponding to the three C282Y genotypes (wild-type, heterozygous and homozygous for the mutation). As the detection probe was complementary to the mutant sequence, its melting occurred earlier in samples containing the wild-type sequence than in those containing the mutated sequence. The melting temperatures (Tm, mean ± SD) were 59.03 ± 0.30°C for the wild-type allele and 63.97 ± 0.14°C for the mutant allele. Heterozygous samples produced these two melting peaks, as expected. Figure 1B shows the results obtained for H63D and S65C genotype analysis. As with the C282Y probe, the detection probe used for H63D genotyping matched the mutant sequence, so its melting occurred earlier in samples containing wild-type sequence. The Tm values were 62.05 ± 0.35° and 67.46 ± 0.30°C for the wild-type and mutant alleles, respectively. The S65C mutant allele could be identified by a melting peak at 58.24 ± 0.51°C (Figure 1B). An individual heterozygous for both S65C and H63D mutations is expected to present melting peaks at 58.24° and 67.46°C, while an individual heterozygous only for S65C should present melting peaks at 58.24° and 62.05°C. An individual homozygous for S65C mutation but H63D wild-type is expected to present a single melting peak at 58.24°C. Assuming that the H63D and S65C mutations do not occur on the same chromosome (12), the genotype combinations S65C/H63D compound homozygous, as well as S65C heterozygous/H63D homozygous (and vice versa), are not probable. The difference in Tm among H63D wild-type, H63D mutant and S65C alleles was greater than 3°C, allowing accurate determination of alleles.

Even though multiplex PCR with hybridization probes has been used before on the LightCycler for hemochromatosis genotyping (8,9,13), it was performed with two probes

Table 1. Primer and probe sequences used for genotyping the *HFE* gene by quenched-FRET real-time PCR.

Sequences (5'-3')	Tm (°C)
C282Y	
Primer forward: CTGGATAACCTTGGCTGTACC	55.3
Primer reverse: GGCTCTCATCAGTCACATACC	55.1
Probe FRET: CAGAGATATACGT**A**CCAGGTGGAGCA-FAM	61.6
Probe anchor: Cy5-GCCTGGATCAGCCCCTCATTGTGAT-P	64.7
H63D/S65C	
Primer forward: GTCTCCAGGTTCACACTCTC	54.3
Primer reverse: CCATAATAGTCCAGAAGTCAACAG	54.7
Probe FRET: TAMRA-CGTGTTCTATGAT**G**ATGAG**A**GTCGCCG-P	67.1
Probe anchor: GCTTTGGGCTACGTGGATGACCAGCT-JOE	63.7

The underlined bold nucleotides correspond to the polymorphic bases involved in duplex mismatch formation. For C282Y and H63D, the probes are complementary to the mutant allele; for S65C, the probe is complementary to the wild-type allele. P indicates the addition of a phosphate group to prevent extension by *Taq* polymerase from the 3' end of the probe. Tm = melting temperature.

labeled with the same acceptor dye. This is possible only if the different melting points of the two probes are always clearly discernible, which is not always feasible. The primer and probe set employed by Bernard et al. (8) was the only one capable of detecting the three mutations in the *HFE* gene, but with a 1.5°C difference in Tm between S65C and C282Y mutant alleles, which may pose some risk of a false-positive C282Y when multiplexing. The C282Y and H63D detection probes designed for this study were labeled with different fluorophores (6-FAM and JOE), which allowed data acquisition in two different channels. Therefore, it was possible to detect three single base pair mutations (representing six alleles) in one tube, in a single run. The combination of fluorescence detection with melting profiles provided a powerful tool for multiplex SNP detection in *HFE*. Moreover, the use of two different fluorophores avoids the risk of misinterpretation that may arise in multiplex reactions due to very similar Tm values.

The allelic frequencies obtained in the present study were 9.4, 20.0, and 0.6% for the C282Y, H63D and S65C mutations, respectively. Genotype frequencies were as follows: 282 C/C, 86.2%; 282 C/Y, 8.8%; 282 Y/Y, 5%; 63 H/H, 62.5%; 63 H/D, 35%; 63 D/D, 2.5%; 65 S/S, 98.7%; 65 S/C, 1.3%. The compound genotype frequencies were: 282 C/C 63 H/H 65 S/S, 47.5%; 282 C/C 63 H/D 65 S/S, 35%; 282 C/Y 63 H/H 65 S/S, 8.8%; 282 Y/Y 63 H/H 65 S/S, 5%; 282 C/C 63 D/D 65 S/S, 2.5%; 282 C/C 63 H/H 65 S/C, 1.3%. The C282Y allelic and carrier frequencies detected in the present study were higher than the frequencies reported by Brazilian studies published previously (14-16), in contrast to H63D allelic and carrier frequencies, whose values were similar. The reported prevalence of C282Y and H63D carriers in these studies were, respectively, 1.2 and 31.1% (14), and 2.8 and 32.6% (15), in the general population and in healthy controls. The allelic frequency for the S65C mutation found in the pres-

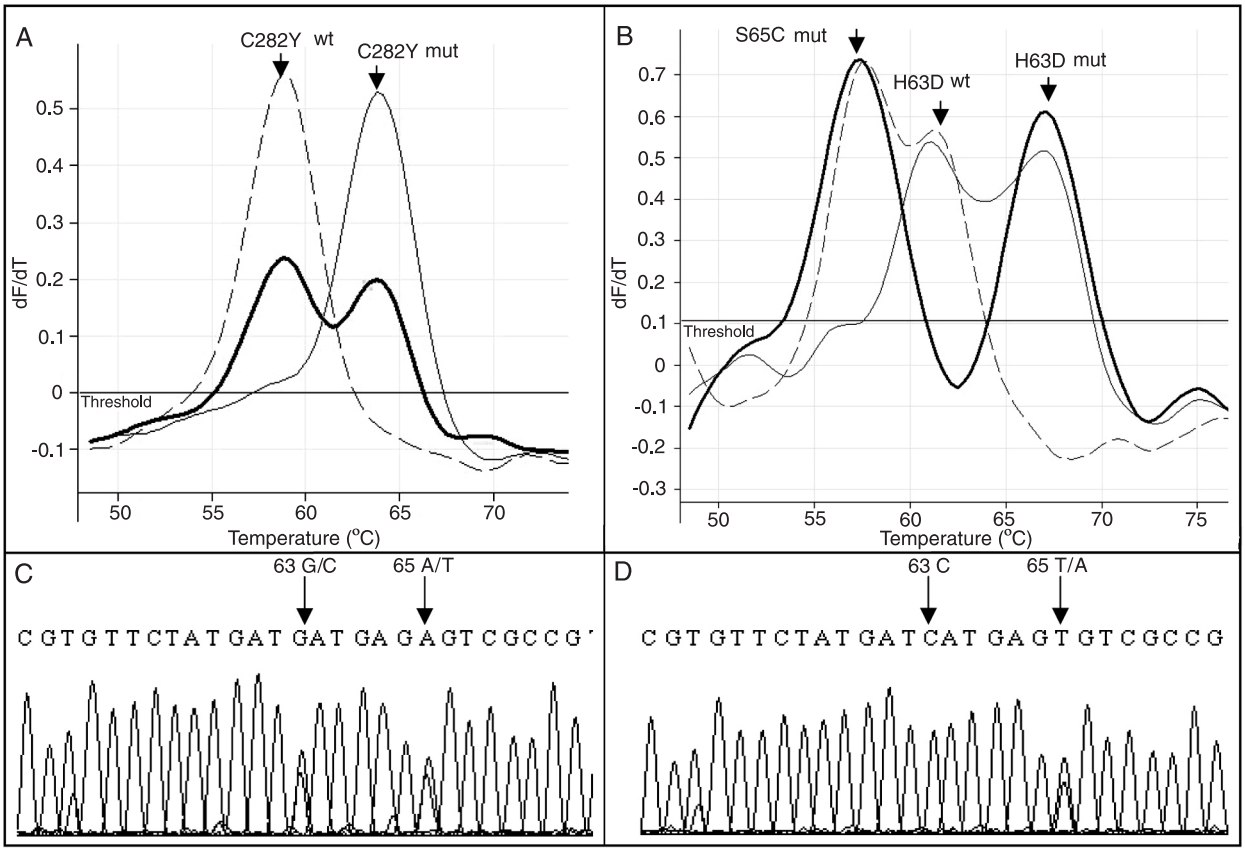

Figure 1. Derivative melting curves (-dF/dT x T°C) and sequencing results showing genotypes. *A*, C282Y wild-type (wt, dashed line), heterozygous (thick full line) and homozygous for the mutation (mut, thin full line). *B*, H63D/S65C compound heterozygous (thick full line), H63D heterozygous/S65C wild-type (thin full line), H63D wild-type/S65C heterozygous (dashed line). *C*, Nucleotide sequence of an H63D/S65C compound heterozygous (SNPs at codons 63 and 65 are indicated by arrows). *D*, Nucleotide sequence of H63D wild-type/S65C heterozygous (SNPs at codons 63 and 65 are indicated by arrows).

ent study was similar to those obtained in a survey with both β-thalassemic carriers and individuals without hemoglobinopathies (0.6 and 0.87%, respectively) (17). It is important to emphasize that the group surveyed in the present study is mainly composed of individuals who present hematological alterations, which *per se* introduces a bias. Therefore, these data should have no epidemiological significance and cannot be extrapolated to the general Brazilian population.

In contrast to C282Y mutation, which is clearly associated with HH, the role that the H63D substitution plays in the development of iron overload disease has been controversial. It seems to increase the risk for HH when coinherited with the C282Y mutation, in the compound heterozygous state, which accounts for 3-5% of cases of HH (18). However, the clinical significance of the other forms of compound heterozygosis, such as C282Y and S65C or H63D and S65C, is still uncertain (19). No compound heterozygotes C282Y/H63D, C282Y/S65C or H63D/S65C were detected among the 80 individuals surveyed for this study. In rare HH cases, none of these known *HFE* mutations are present. Other mutations, possibly not associated with *HFE*, might contribute to the development of hemochromatosis.

All 80 genotypes obtained both by conventional PCR-RFLP and quenched-FRET real-time PCR were in full agreement. However, RFLP methods require multiple manual steps and are time-consuming while real-time PCR combines PCR amplification, mutation detection and laboratory report into a single step. As a consequence, there was a significant reduction in the total time needed to process the samples comparing real-time PCR (2.5 h) to PCR-RFLP (12 h), since some steps such as enzyme digestion, electrophoresis and staining with ethidium bromide were eliminated. Additionally, the method described here did not suffer failure of amplification of the H63D wild-type allele when the S65C mutation was present, a phenomenon previously reported by Koeken et al. (20) to occur with the method by Stott et al. (5).

The quenched-FRET real-time PCR method also offers some advantages over the allele-specific real-time PCR, since the latter is subjected to false-homozygote results in the presence of a mutation in one of the probe annealing sites. Since real-time PCR based on FRET probes employs the same oligonucleotides for the detection of both wild-type and mutant alleles, failure in any oligonucleotide hybridization would affect the whole reaction, avoiding erroneous results. Additionally, the costs involved in allele-specific probe assays are equivalent to those based on the FRET principle, since both require the use of two fluorescent probes.

DNA sequencing analyses of three samples harboring distinct genotypes confirmed the results obtained by real-time PCR genotyping, which proved to be an accurate, rapid and cost-effective diagnostic assay.

Our findings demonstrate the usefulness of quenched-FRET real-time PCR for the simultaneous detection of three mutations in the *HFE* gene, which allows reduction of a significant amount of time in sample processing compared to the PCR-RFLP method, eliminates the use of toxic reagents, such as ethidium bromide, reduces the risk of contamination and errors in the lab and allows process automation.

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